The BioFlip Microcytometer

Final Technical Report:
July 2000–September 2004
Contract No. MDA972-00-C-0029
Data Item 0002AC

September 10, 2004

Prepared for:

Defense Advanced Research Projects Agency
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UNCLASSIFIED

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Sponsored by:

Defense Advanced Research Projects Agency
Microsystems Technology Office
BioFlip Microcytometer
ARPA Order No. K425/01/01
Issued by DARPA/CMO Under Contract No. MDA972-00-C-0029

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	10 Sept. 2004	Final Technical Report— July 2000–September 2004

4. TITLE AND SUBTITLE	5. FUNDING NUMBERS
The BioFlip Microcytometer	MDA972-00-C-0029
6. AUTHOR(S)	
Dr. Aravind Padmanabhan	
7 PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	**8. PERFORMING ORGANBIZATION REPORT NUMBER**
Honeywell Laboratories 12001 State Highway 55 Plymouth, MN 55441-4799	C04182
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	**10. SPONSORING/MONITORING AGENCY REPORT NUMBER**
Defense Advanced Research Projects Agency Microsystems Technology Office 3701 North Fairfax Drive Arlington, VA 22203	

11. SUPPLEMENTAL NOTES

The views and conclusions contained in this document are those of the authors and should not be interpreted as representing the official policies, either expressly or implied, of the Defense Advanced Research Projects Agency or U.S. Government.

12a. DISTRIBUTION/AVAILABILITY STATEMENT	12b. DISTRIBUTION CODE
Distribution Statement A: Approved for Public Release; Distribution is unlimited.	

13. ABSTRACT (Maximum 200 words)

This four-year program successfully demonstrated a point-of-care (POC) integrated scattering and fluorescence flow cytometer capable of counting and classifying white blood cells from a whole-blood sample input. The instrument development and overall system testing tasks on this project were performed by Honeywell and the disposable cartridge development was led by Micronics. The revolutionary miniaturization of a cytometer was enabled by the core technology strengths of Honeywell and Micronics. As part of this project, the Honeywell-led team has demonstrated three-part differentiation of white blood cells using a 6 in. x 6 in. x 4 in., 3-lb light-scattering-based cytometer. The cytometer is composed of a handheld instrument and credit-card-sized disposable analysis cartridges. The system has the following features: automated sample preparation on the cartridge, red-VCSEL-array-based electronic self-alignment, highly miniaturized multi-channel pumping system, and custom-developed electronics and graphic user interface. In addition, the team has demonstrated a highly miniaturized integrated two-color light-fluorescence based cytometer. Repeatable counting and classification of two-color bead mixtures was demonstrated in this cytometer. This handheld, easy to use instrument can be modified for the detection of biowarfare agents in environmental samples.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Cytometer, Microfluidic Cartridge, Point of Care Instrumentation, Closed-loop pumping system, VCSEL array based optical system, White Blood Cell Differentiation, CD4/CD45 assay, light scattering, light fluorescence.	16. PRICE CODE

17. SECURITY CLASSIFICATION	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

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Section 1. Program Objectives

1.1 Instrument

The major objectives of the four-year program were twofold:

- **Develop Palm-Size Scattering Cytometer (Baseline Program):** Develop a fully integrated, highly miniaturized scattering and fluorescence microcytometer capable of indicating viral and bacterial infections based on a quick measurement of lymphocyte and neutrophil counts.
- **Develop Miniaturized Integrated Scattering and Fluorescence Cytometer (Add-On Program):** Develop a fully integrated, highly miniaturized scattering and fluorescence microcytometer capable of identifying and counting subclasses of white blood cells based on fluorescence measurements.

The secondary objectives were to:

- Demonstrate white blood cell counting and classification through precise light scattering measurements performed with smart optics, including arrays of vertical-cavity surface-emitting lasers (VCSELs), photodiodes, and microlenses;
- Demonstrate a highly miniaturized closed-loop fluid driver system that can generate the range of flows necessary to perform sample preparation and cytometery measurements on the cartridge.
- Demonstrate structures, techniques, and materials for inexpensive manufacturing of the microcytometer system with disposable cartridges, sample collectors, and instrument body housing.
- Demonstrate maintenance-free optical scattering module with self-alignment capability (based on red VCSEL arrays) capable of rapid (~ 30 seconds) counting and classification of lymphocytes and neutrophils.
- Demonstrate fluorescence optical module capable of counting and detection of specific targets in the sample (WBC, blood-based proteins or specific antigens).

1.2 Cartridge

The cartridge development task objectives were:

- **Demonstrate disposable analysis cartridges (credit card size) for performing both scattering and fluorescent cytometry**. These cartridges contain sample collectors, reagent storage reservoirs, liquid flow sensors, and microflow channels for cytometry measurements.
- Develop a method to lyse red blood cells (RBCs) on the cartridge.
- Perform fluidic modeling of the cartridge.

- Test the fluidic card with whole blood on miniaturized cytometer platforms.
- Develop and refine the method to stain (attached tagged antibodies to blood cells) white blood cells (WBCs) on the cartridge. And integrate the method onto a cytometric fluidic card. Test the integrated card with whole blood using Honeywell instrument.

Section 2. Technical Problems

2.1 Instrument

A short summary of the technical challenges encountered in the development of the scattering and fluorescence cytometer are given below. *It must be noted that successful technical solutions to each of these challenges was developed by the Honeywell-led team.*

- Achieving the required control and stability of the flow rates in a pressure-driven system based on direct flow rate measurement and closed-loop pressure control.
- Achieving the required precision in the metering of the sample volume in a pressure- as opposed to a volume-controlled driving system.
- Achieving a reproducible and satisfactory alignment between the optical components on the instrument and the flow path on the disposable fluidic chip.
- Obtaining necessary scattering data for cell classification in the proposed optical arrangement.
- Ensuring good, long-term seal for the on-chip reservoirs and waste container.
- Achieving a good pneumatic/hydraulic coupling between instrument and cartridge.
- Achieving light tightness for the optical module of the miniaturized fluorescence cytometer.
- Achieving high efficiency/sensitivity of fluorescent light collection on the detection side of the fluorescence optical module.
- Embedding die-level liquid flow sensors on the disposable fluidic cartridge.

2.2 Cartridge

The specific card development challenges that were addressed over the course of the project are as follows.

2.2.1 Scattering Cytometer Cartridge

2.2.1.1 Leakage—For the complex, prototype laminate lab cards being developed, leakage was seen around both the embedded Honeywell flow sensors and the cytometer window. The card design was modified by moving other on-card features, namely the focusing chamber, which resolved most of the cytometer window leakage problems [1]. The team has identified commercial (as opposed to prototype) manufacturing processes to seek to ensure sealing of the card when produced in volume.

In the fully integrated card (sample loop, reagent storage, flow sensors, lyse loop, cytometer; card model #HWC-15), leakage occurred in the thin film laminate constructed prototype lab card between the sheath reservoir, Honeywell flow sensor and cytometric window components on card. The amount of adhesive using in the laminate process between the reservoir and the

individual component cutouts was insufficient to maintain a seal when pressure was applied to the sheath fluid. This was resolved by card model #HWC-28, where the shape of the sheath reservoir was changed, in order to provide greater distance between it and the cutouts.

2.2.1.2 Wetout—"Wetout" is the term used to denote the first fluidic filling of a channel or reservoir on a lab card. The type of material the channel is made of, as well as the shape of the space to be filled must be addressed in order to solve wetout problems. Wetout problems are generally seen as a trapped air bubble or unfilled spaces against the walls of a channel in the initial designs of a new lab card.

For capillary channels it is important to have high energy surfaces [2]. Micronics applied plasma treatment in the laminate card construction process for all sample and lyse loops which improves wetout, although this treatment does have a shelf-life that can be addressed in further commercial development [3].

The benefits of plasma treatment do not carry over for filling larger voids, such as the lyse injector and focusing chamber on card. These structures required that fluid fill both the top and bottom flat surface holding the sample channel. Since the two sides do not fill at the same rate, the side that fills faster stops at the end of the sample channel. This does not happen if the surfaces have been plasma treated. The result is that the side that wets out first flows backward on the opposite side, trapping air and making further flow on that side impossible [1].

The team demonstrated that in order to minimize wetout failures, various card layers must be selectively plasma treated. Eliminating wavy cuts is also important, to reduce areas where bubbles can be trapped.

2.2.1.3 Reagent Storage—The main challenge to reagent storage on card is encapsulating the reagent with little or no fluid loss. The team explored various methods of sealing the inlet and outlet ports. All seemed to work equally well when care was taken to adequately seal the ports.

A further issue with reagent storage is the formation of air bubbles within the reservoirs over time. The team found that degassing the fluids before filling the reservoir eliminated this problem [4].

2.2.1.4 Tendency of Blood to Clump in Lyse Injector—Whole blood will clump in the lyse injector if the sample flow rate is not high enough. The minimum flow rate is 0.5 µl/s [5]. Another cause of clumping is pushback of lyse fluid into sample fluid, which was observed with Alpha platform. This was however not a problem with Honeywell's miniaturized pumps.

2.2.1.5 Prototype Production Issues—Some of the prototype production issues were mentioned in the previous sections. These include surface treatment, straight cuts, and adequate surface area for adhesion. Micronics identified methods and means to provide prototypes for testing as well as continuing to evaluate transitional methods for commercial volume production.

It is important to note that the specifications for the lab card for the project required initial development of a complex, three-dimensional structure in which each fluidic circuit was independently tested prior to integration. As such, this card through its various configurations required a manually intensive prototype production process that can contribute to alignment

errors, material cleanliness issues, scratching of materials, and human error. All of these issues are addressable in commercial production methods.

It is expected that once the card design is finalized, manufacturing will progress from the prototype process (laser cut), into the design verification stage (die cut), and finally into injection molding. Injection molded cards will resolve these manufacturing issues.

2.2.2 Fluorescence Cytometer Cartridge

2.2.2.1 Materials—TOPAS (COC) was used in the cytometer window because it has low autofluorescence and good optical properties. This material was very difficult to cut with a CO_2 laser, due to poor CO_2 wavelength absorption with the COC material.

The TOPAS material doesn't thermally ablate when cut with the CO_2 laser; instead it melts and leaves material as an uneven ridge along the cut ("potato chipping") and molten material blowing back across the uncut sheet, leaving speckles and streaks (see Figure 1). Micronics employed new cutting methods to minimize material deposits on the optical window portion of the card.

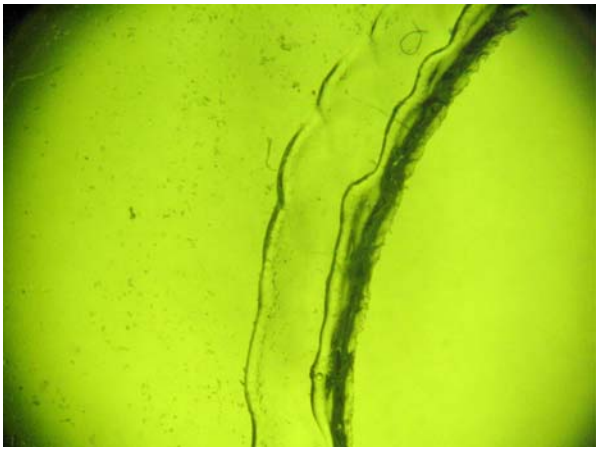


Figure 1. Photo of TOPAS Laser Cut Showing "Potato Chipping" and Material Blowback Causing Speckling

The ridge was pronounced enough that the adhesive was held above the main card surface, resulting in gaps in the lamination. This was especially true around the cytometer nozzle. To rectify this, Micronics cut the layers in a different fashion, forcing the edges to be on the outer surfaces of the cytometric nozzle.

Section 3. General Methodology

The key features of the various generations of microcytometers built under this program are:

- **Miniaturized optical subsystem** (red VCSEL array, lenses, photodetector array);
- **Miniaturized fluid driver subsystem** (microvalves, flow sensors, pressure source);
- **Disposable microfluidic analysis cartridge** (cytometer channel, lysing channel, reagent reservoirs, sample acquisition and storage);
- **System electronics** (electronics for various subsystem and for overall system);
- **Graphical user interface**.

3.1 Instrument

This section discusses the various aspects of the development of the instrument for both the scattering and fluorescence cytometers.

3.1.1 System Design

A fundamental aspect of the system design was the partitioning of the microcytometer system into the disposable fluidic card and the non-disposable instrument body. The development of a highly miniaturized cytometer relies on the integration of three advanced technologies that are key to blood cell analysis, as shown in Figure 2:

- **Low-power microsensors and microactuators** for closed-loop flow sensing and control,
- **Fluidic processing & sample preparation** based on miniaturized fluidic cartridges, and
- Optical light scattering/fluorescence based detection using **arrays of solid-state lasers/detectors and micro-optics**.

We have identified the ideal components to achieve the integration of sample collection, sample processing, fluid delivery, and optical detection to yield a compact microcytometer. The Honeywell-Micronics team brings together leaders in each of these fields, providing a unique opportunity for the integration of advanced and complementary technologies into a revolutionary new product.

The currently available commercial cytometer systems are limited to relatively large, benchtop-type instruments that remain in central laboratory environments due to their complexity. Miniaturized systems would put the important analytical capabilities of a cytometer closer to the individual, providing a rapid alert and potential therapeutic intervention capability for hematologic abnormalities.

For both safety and CLIA waiver reasons it is essential that all materials coming into contact with blood be disposable. Thus the sample introduction system and all the fluid handling system is part of the disposable fluidic card in brassboard cytometer version 2.0. The blood is introduced into the disposable chip where it is diluted and lysed. The diluted/lysed blood is then surrounded by the sheath fluid and focused into single file for analysis. The fluid driving system, the optical detection system, and the electronic components are on the instrument body into which the disposable chip is inserted. The microfluidic channels, reagent reservoirs, and the waste fluid reservoir are on the disposable card. Figure 2 shows a photograph of the brassboard cytometer version 2.0 (called POC cytometer) that was built and characterized in the third year of the project. Figure 3 shows the technology development roadmap.

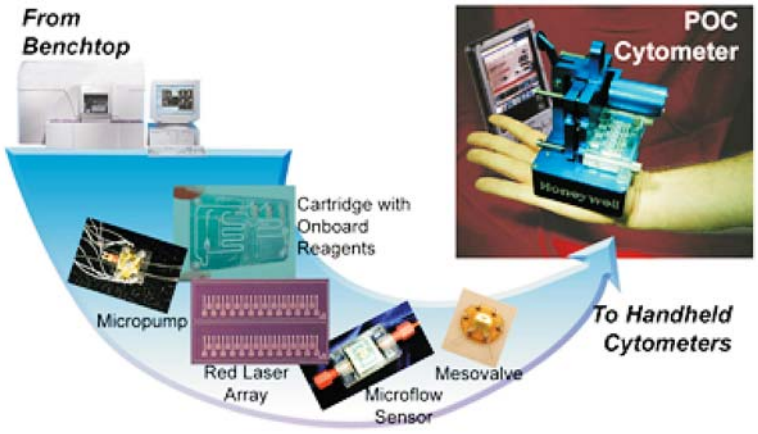


Figure 2. Schematic illustrating the MEMS/Microphotonics/Microfluidics technologies that allowed the Honeywell-led team to perform revolutionary miniaturization of a desktop cytometer instrument into a handheld analyzer.

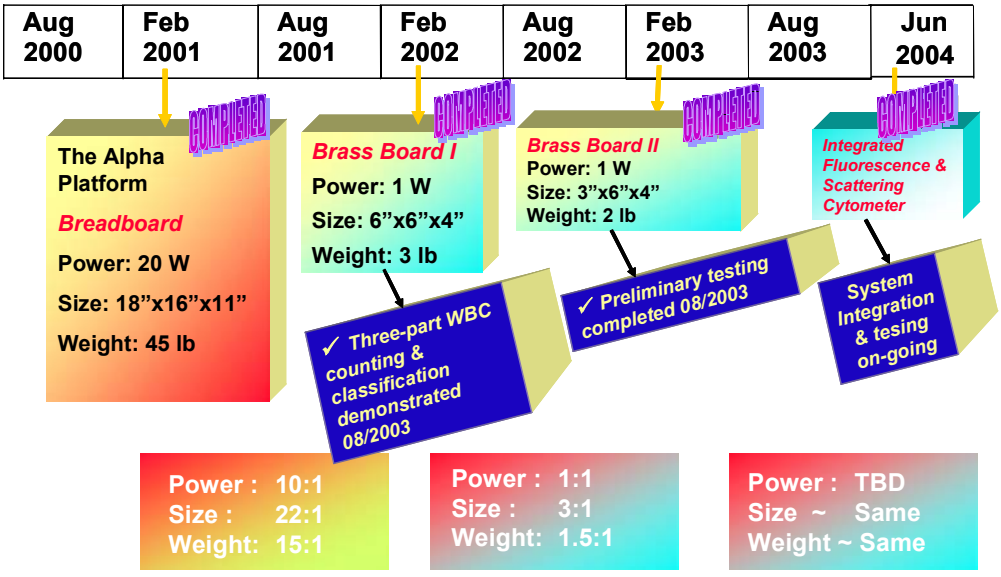


Figure 3. Technology development roadmap for the 4-year DARPA Project

3.1.2 Fluidic Cartridge Design and Manufacturing

The main objective of this task was to develop a credit card sized size disposable fluidic card for doing 3-part differentiation of white blood cells (Scattering Cytometer) and for performing 2-color fluorescence assays (Fluorescence Cytometer). Details of the card design, card operation and test results are provided in section 3.2.

3.1.3 Fluid Driver Subsystem

The pumping system in commercially available hematology analyzers and flow cytometers is based on volume-controlled flow generated by syringe pumps that are driven by stepper motors. Such systems are precise but bulky/power hungry and not suitable for use in POC instruments. As part of this project, Honeywell has developed a new miniaturized pressure-driven (as opposed to volume-driven) pumping system that operates in a closed loop. A photograph of a three-channel version of such a system is shown in Figure 4. The operating principle of such a pump is as follows: a high-pressure source of air is generated using a micropump. Lower and precisely controlled pressures are generated from this high-pressure source by using arrays of miniaturized valves. The valves are used in a closed-loop configuration with micro flow sensors mounted in each flow path to ensure the desired flow rate for each flow channel. Several generations of such pumps have been built, characterized, and shown to work well. Figure 4b shows the precise flow rates that can be achieved with this technology. The high accuracy in the control of the flow rate of the various reagents and blood sample implies a high accuracy for the measured counts of blood cells for the scattering and fluorescence cytometers. It must be noted that a 3-channel pump was used in the two generations of the scattering cytometer while a 4-channel pump was used in the fluorescence cytometer.

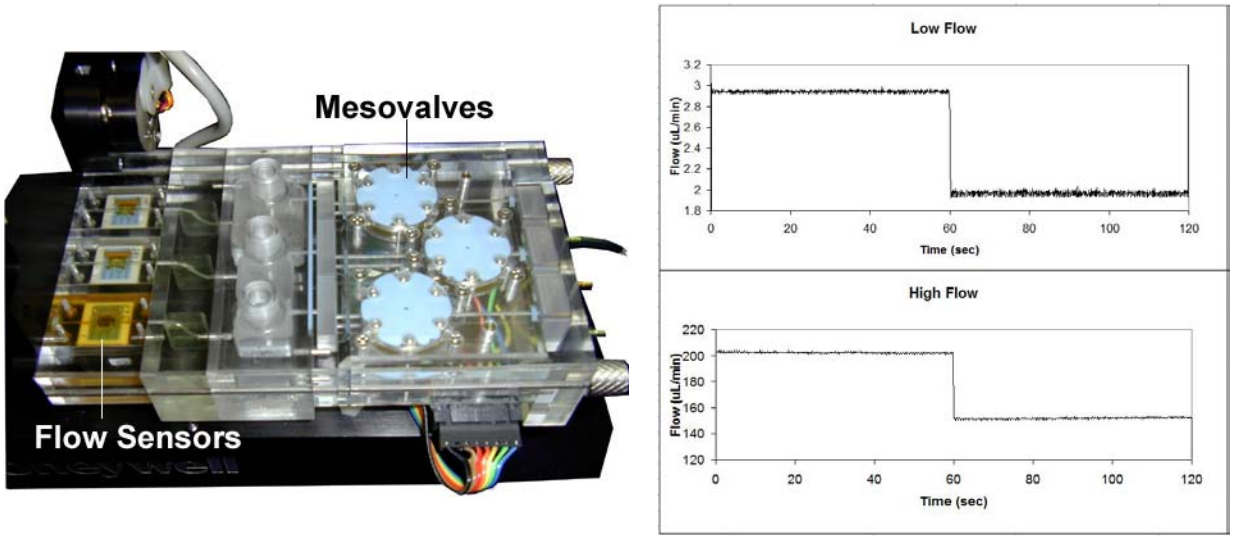
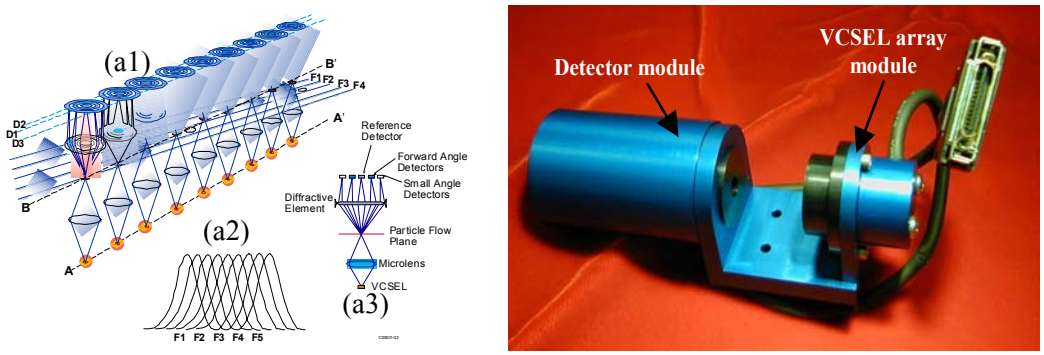


Figure 4. (a) Photograph of a three-channel miniaturized closed-loop pumping system that is 5 in. x 6 in. x 4 in. in size and consumes 150 mW power. This technology will be modified for use in the POC cytometer to be developed under this program. (b) Data showing highly precise (1% accuracy) control of the flow rates of two channels of this pumping system in the flow ranges of 2–3 µL/min and 150-200 µL/min.

3.1.4 Optical Subsystem

In flow cytometry, the optical alignment of the focused laser spot with the flow path of the particle to be analyzed is critical, and most instruments require periodic realignment and maintenance, typically performed by qualified personnel. The alignment problem is exacerbated by the variability in the core location in a low-cost, disposable plastic cartridge. We have demonstrated a novel solution to this alignment problem, based on Honeywell's red (670 nm) Vertical Cavity Surface Emitting Laser (VCSEL) arrays, as shown in Figure 5A. The single laser used in conventional cytometers is replaced with a linear array of VCSELs as that provides: (a) a means of determining the exact path of a particular cell (in the core flow); (b) a means of actively locating the cytometer flow channel; and (c) a means of actively locating the core flow within the cytometer flow channel. Repeatable operation of this electronic self-alignment scheme was demonstrated.



(a) *(b)*

Figure 5A. (a) (a1) The VCSEL array optical subsystem concept showing a linear VCSEL array AA' and focusing microlenses form a line BB' of focused spots in the particle flow plane. (b) Photograph of the compact (5 in. x 2 in.) integrated optical module.

The optical approach for the two color fluorescence cytometer is defined as one where parallel channels of dichroic beam splitters and bandpass filter paths are used in the fluorescence detection leg. Figure 5B shows the optical layout for the parallel approach. The source leg is comprised of a source whose wavelength is blue 488 nm to illuminate the flow channel. The Forward Angle Light Scatter (FALS) detection leg is shown with a PMT detector with bandpass filters allowing for the measurement of the scatter light at the source wavelength. The scatter signal from the FALS detector when plotted against the fluorescence signals, lets the system identify tagged antibodies which do not have an antigen on them, resulting in improved sensitivity of detection. Additional dichroic beamsplitters are used to separate the two fluorescent colors which are detected by 2 PMT detectors. This parallel approach results in a simple detection readout based on proven approaches used in most large bench top commercial cytometers.

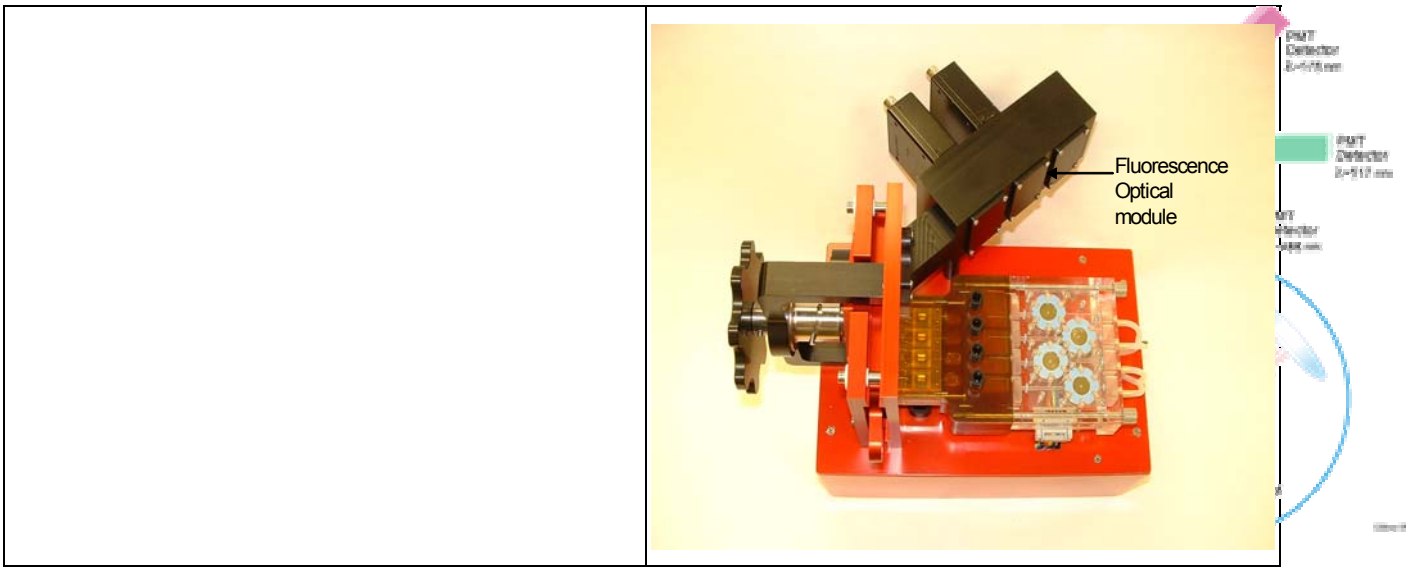


Figure 5B. Schematic diagram and photograph of the two-color fluorescence optical module.

3.1.5 Signal Processing and Cell Identification

The cytometer data acquisition system (functional bloc diagram shown in Figure 6) consists of the signal conditioning and analog-to-digital (A/D) conversion electronics necessary to adequately process optical information from a hydrodynamically focused core of diluted whole blood. An important feature of the electronics system is the multiparameter analysis enabled by the VCSEL arrays. Multiparameter analysis is highly desirable in cytometry to improve separation between classes of blood cells since more scatter angles provide a higher dimensional parameter space. It must be noted that for the fluorescence cytometer an off-the-shelf data acquisition electronics was used. This electronics has the appropriate footprint to fit into the electronics box of the miniaturized instrument.

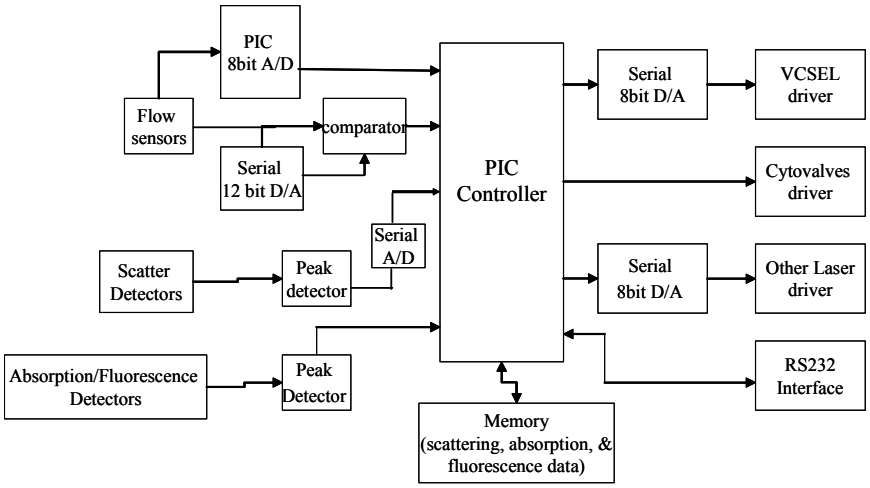


Figure 6. Functional block diagram of the electronics used for the various generations of brassboard cytometer built under this program

3.1.6 System Software

The software for the microcytometer accomplishes three tasks: real-time control (sensor monitoring, pump control, valve control, data acquisition, all calibration functions, and all of the sequencing and timing related to these operations); data analysis and reduction (algorithms to correlate and reduce the data to separate and count white blood cells); and interface control (interactions with the user, another computer, or with a data storage and retrieval system). Figure 7 shows a photograph of the custom graphic user interface developed as part of this project.

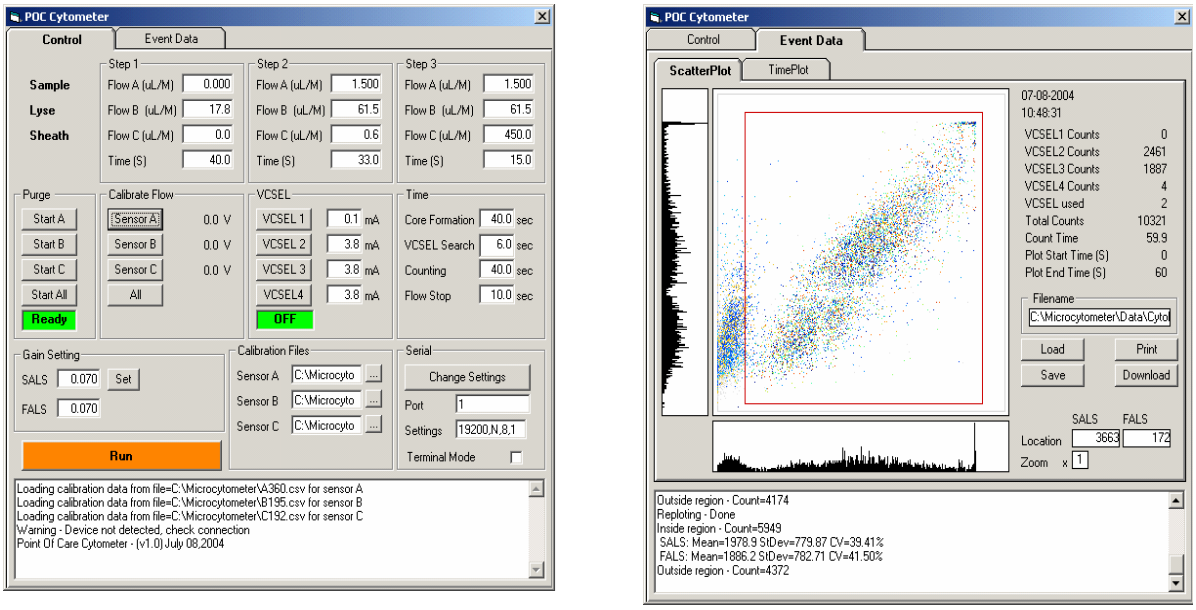


Figure 7. Photographs of the custom graphic user interface developed for the instrument

3.2 Cartridge

Microfluidic cards are thin-film laminates built of several layers of individually cut fluidic circuits. Although each layer may be manufactured quickly and relatively inexpensively, the lamination process yields complex three-dimensional microfluidic structures. This allows the design, for example, of three-dimensional hydrodynamic focusing channels for cell analysis and of multiple separate circuits with intersecting channels on a single card.

The disposable cards built as part of this project are credit-card-sized with fluidic elements ranging from 100 µm to a few millimeters. The laminates are composed of thin plastic sheets, ranging from about 10 µm to a few hundred micrometers thick. The layers are bonded using adhesives. Some internal surfaces of the laminates are chemically treated to change their wettability. Rapid prototyping process allows the design and testing of new microfluidic card designs in as little as 24 to 48 hours. This offers a significant advantage over other microfabrication processes such as silicon microfabrication, micro-injection-molding, or

chemical etching processes. Integrated microfluidic cards, such as those developed under this project, are operated by external pumps.

3.2.1 Fluidic Subcircuit Design

The Micronics team has developed a systematic approach to producing integrated polymeric lab cards for multiple product applications. Micronics reduces the multiple microfluidic functions for a given application into the simplest form (called subcircuits).

3.2.2 Material Selection, Surface Treatment, Adhesives

Material selection is pivotal in the lab card development process to ensure a properly functioning card. The fact of high surface to volume ratios means that plastic surfaces can effect the operation of a given assay. There is no universal plastic that meets the needs of every card design given the breadth of possible applications, chemistries and other components that may be integrated for optimal performance. Rather, Micronics employed a variety of plastics and adhesives that may be balanced with the specifications of the desired card's functionality. An example of the importance of material selection is indicated below, in which selections of plastics were evaluated for absorption of scatter laser light from particles in the fluorescence cytometer project. In this example, the team assessed plastic film candidates for material opaqueness suitable to 488 nm (Figure 8).

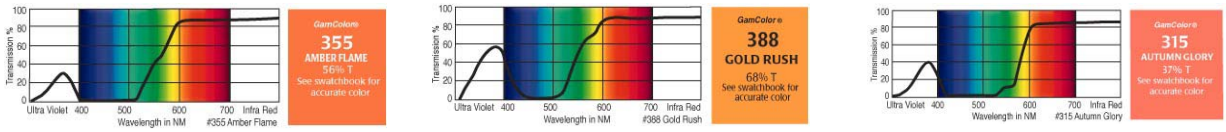


Figure 8. Absorbance of Polyester Film with Various Pigments

Another example deals with liquid reagents being stored on the plastic microfluidic lab card. The material properties of the plastics must be matched to the gas permeability ($cm^3/100$ in2·24 hr·atm/mil @ 25 C) of plastic films. The barrier properties of the films play a critical role in preventing liquids from drying out (H_2O migration), as well as preventing pH drift (minimizing migration of O_2 and CO_2). Thus, material selection is of particular importance to each project given an objective that the commercial disposable card be stable at ambient temperature for up to one year and be capable of use in remote regions where robust and ruggedized designs are essential.

3.2.3 Design Control

Micronics employed design control guidelines throughout its lab card development efforts. All designs receive an independent bar code and are tracked for materials used, design features, test results, and other functional issues important to subsequent design iterations or final production of commercial lab cards. This section discusses the various aspects of the development of the cartridge for both the scattering and fluorescence cytometers.

3.2.4 Scattering Cytometer Cartridge

3.2.4.1 Cytometer Subcircuit—The Microcytometer is a patented Micronics design [10, 11, 12, 13, 14], which was integrated into this system. The original cytometer subcircuit is shown in Figure 9 and the final cytometer subcircuit (referred to as a two-channel card) is shown in Figure 10. Various nozzle and focusing chamber configurations were evaluated.

3.2.4.2 Lysing Subcircuit—The lyse injector and loop is a patented Micronics design [14], which was integrated for use in this system. The original lysing subcircuit is shown in Figure 11. The final configuration is part of the integrated cytometer/lyse card detailed in the next section.

3.2.4.3 Integrated Cytometry and Lysing Subcircuits—The cytometer and lyse injector subcircuits were combined onto one fluid card, known as a three-channel card. The original lyse-cytometer subcircuit is shown in Figure 12. The final configuration is shown in Figure 13.

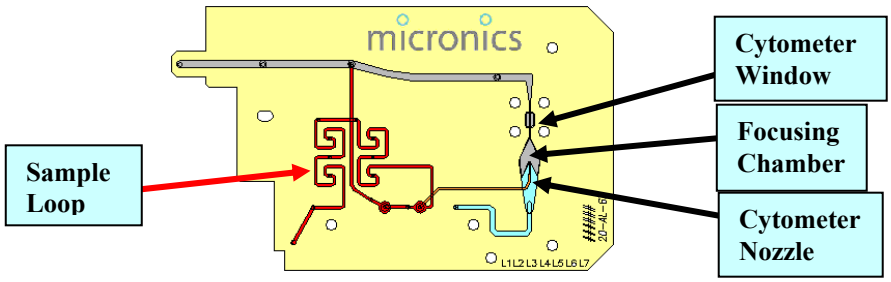


Figure 9. Cytometer Subcircuit (Compatible with Alpha Platform)

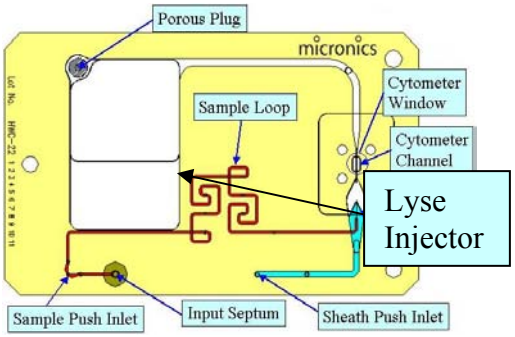


Figure 10. Final Cytometer Subcircuit Design (Compatible with Honeywell Brassboard Version 1.0)

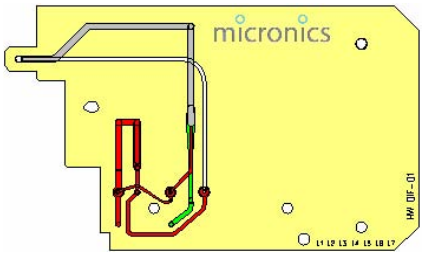


Figure 11. Lysing Subcircuit (Compatible with Alpha Platform)

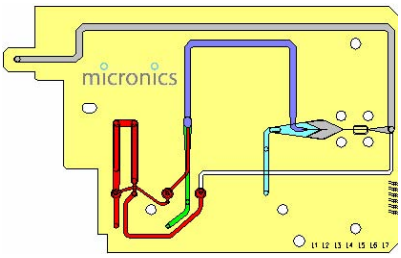


Figure 12. Combined Cytometer and Lyse Subcircuit Design (Compatible with Alpha Platform)

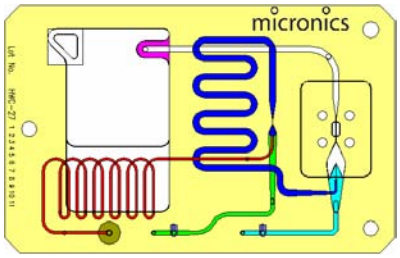


Figure 13. Final Integrated Cytometer and Lyse Subcircuit Design (Compatible with Honeywell Brassboard Version 1.0)

3.2.4.4 Sample Acquisition Subcircuit—Types of circuits tested include:

- Passive capillary action draws sample into storage loop
- Active positive pressure applied by external syringe
- Active negative pressure drawing using a bellows activated by finger pressure.

3.2.4.5 Reagent Storage Subcircuit—Types of circuits tested :

- Large reservoir in acrylic with taped off ports
- Long, thin fluid channel with taped off ports
- Long, thin fluid channel with valving to direct fluid flow
- Long, thin fluid channel with ball valve to close off fluid flow
- Small reservoirs in acrylic with ball valve

3.2.4.6 Integrated Card with Sample Acquisition, Reagent Storage, Lysing, and Cytometer Subcircuits—See Figure 14.

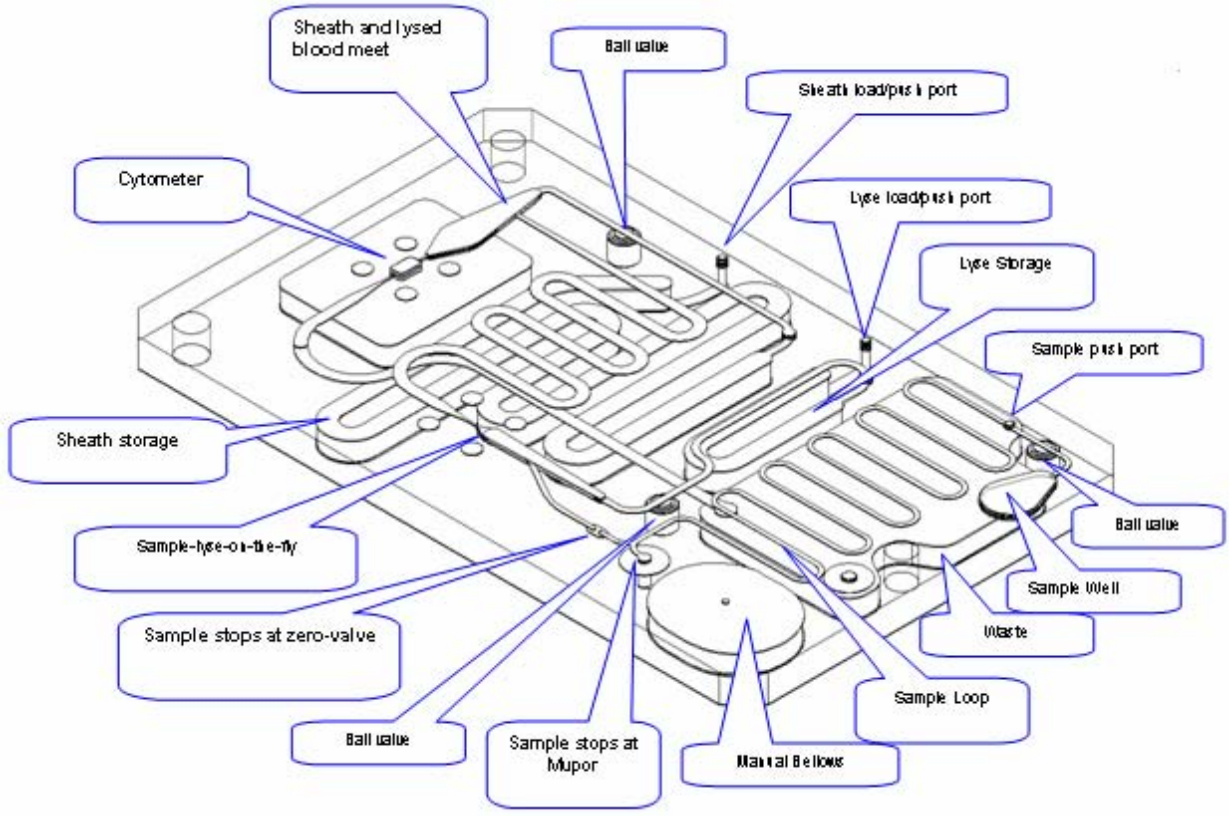


Figure 14. Integrated Card Design with Reagent Storage, Sample Acquisition Added to Three-Channel Card

3.2.4.7 Embedded Flow Sensor—Sensors were first embedded in a card with no other circuits. Then the sensors were added to a three-channel card (Figure 15).

3.2.4.8 Fully Integrated Card—All Subcircuits—See Figure 16.

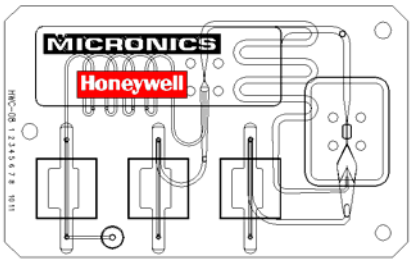


Figure 15. Honeywell Flow Sensors Embedded in Three-Channel Card Design

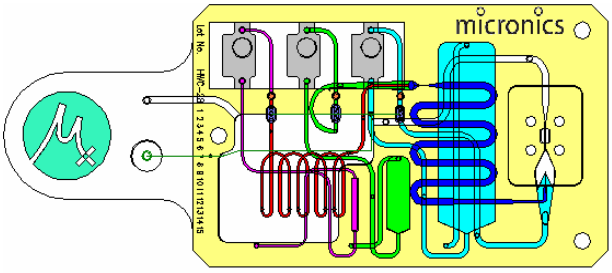


Figure 16. Fully Integrated Card with embedded flows sensors and on-board reagent reservoirs designed for doing 3-part differentiation of white blood cells

3.2.5 Fluorescence Cytometer Cartridge

3.2.5.1 Staining Subcircuit—See Figure 17.

3.2.5.2 Staining and Lysing Subcircuit—See Figure 18.

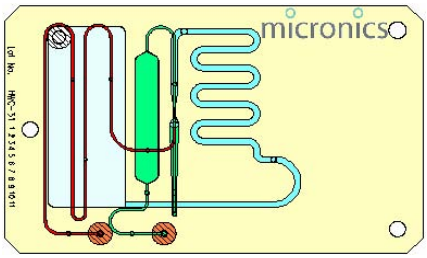


Figure 17. Staining Subcircuit, with Storage for Antibodies on Lab Card

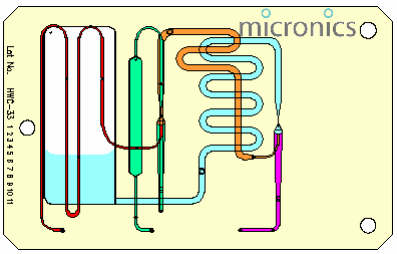


Figure 18. Integrated Staining and Lysing Subcircuits on Lab Card

3.2.5.8 Final Four-Channel Card Combining Cytometer, Lysing, and Staining Subcircuits—See Figure 19.

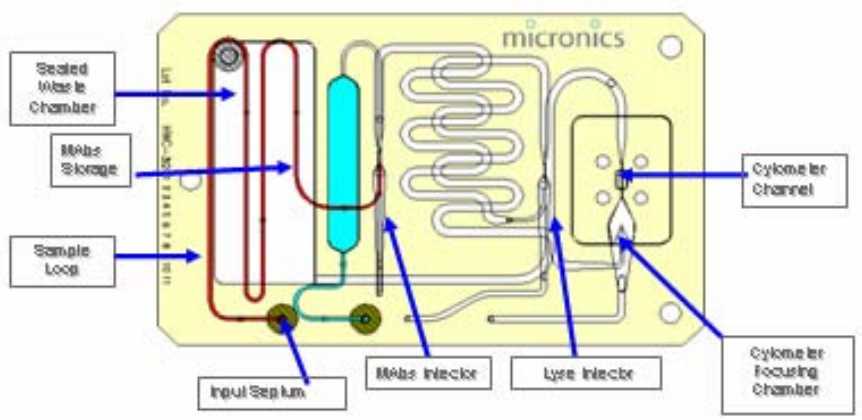


Figure 19. Integrated Fluorescence Card Design with On-Card Staining (tagged antibody-antigen binding), red blood cell Lysing, and Cytometer Subcircuits

Proof-of-Concept Two Color Assay: CD4/CD45 Assay For HIV Patient Monitoring

Why CD4/CD45 Assay was chosen:

- ✓ Enables ease of miniaturized instrument development: CD4/CD45 assay is a standard assay which can easily be verified on commercial cytometers
- ✓ Allows for easy modification to other tests of interest to the Military: miniaturized instrument developed for CD4/CD45 test can be easily modified for other types of tests, i.e., detection of BW agents in different sample types. CD4/CD45 assay is less hazardous to develop and allows the team to optimize the assay protocols on a miniaturized instrument and disposable card.
- ✓ Commercial Significance: CD4/CD45 assay is the most common assay performed on commercial fluorescence cytometers

Clinical Significance of CD4/CD45 Assay

- ✓ CD4 and CD45 are target cells of interest for HIV patients on Anti Retroviral Therapy (ART)
- ✓ CDC and WHO have released guidelines for ART monitoring using CD4 percentages
- ✓ CD45 is added to differentiate WBCs and lymphocytes
- ✓ CD8 adds additional information, but is not required by CDC or WHO guidelines
- ✓ CD8 and other "CDXX" parameters can easily be added into our instrument in the future

It must be noted that the instrument can be used for any other two color assay, e.g., detection biowarfare agents in environmental samples. The on-card sample preparation will however need to be modified to adapt the instrument for this assay.

Section 4. Technical Results

4.1 Scattering Cytometer

The program has demonstrated the following key results:

- Demonstrated three-part white blood cell (WBC) differential using the first-generation brassboard cytometer. Total WBC counts measured with Honeywell personal flow cytometer are within 6% of measurement made by commercial benchtop flow cytometer.

- Demonstrated repeatable counting & classification of bead mixtures (2, 4, 5, 6, 10 μm diameter). The absolute counts of the beads of various diameters were demonstrated to be in close agreement with the theory.

- Demonstrated core flow formation using whole blood on the second-generation brassboard cytometer with flow sensors and reagents on the cartridge.

- Demonstrated repeatable operation of the lyse-on-the-fly procedure using whole blood input to the analysis cartridge.

- Program has delivered on all its initial goals and allows for early technology transfer of Brassboard-like systems.

4.1.1 Fluid Storage Results

We demonstrated lysing fluid storage on the cartridge:

- 180 μl of reagent was introduced to the card through the septum. The channel was taped off at the other end.
- Liquid loss is linear over time, with 7.3 μl lost over 7 days at room temperature, as determined by gravimetric measurement.

We demonstrated sheath fluid storage on the cartridge:

- 1 ml of reagent was introduced to a 1/6-mm-thick card.

4.1.2 Optical & Fluid Driver Subsystem Results

Optical Subsystem

- Demonstrated repeatable operation of a red VCSEL array based miniaturized optical sybsystem (5 in. x 2 in. form factor) capable of electronic self-alignment. This optical subsystem is compatible with both the first and second-generation brassboard cytometers.

- We demonstrated active finding of the cytometer channel, as well as active finding of the core flow in the cytometer channel.

Fluid Driver Subsystem

- We demonstrated repeatable operation of two generations of fluid driver systems. The first-generation system has flow sensors and reagents outside the cartridge, and the second-generation system has them on the cartridge.
- We demonstrated closed-loop flow control on three different flow ranges for both the first- and second-generation fluid driving system.
- We demonstrated embedding and calibration of MEMS liquid flow sensors into fluidic cartridges.
- Finally, we demonstrated operation of new polymer microvalves and associated modules.

4.1.3 Instrument Testing Results – Bead Mixtures and Whole Blood Testing

Summary of test results on the palmsize scattering cytometer is as follows:

- We demonstrated repeatable counting and classification of single and multi-bead mixtures. The scatterograms were in good agreement with theoretical predictions based on Mie scattering theory. The following pages report on typical measurement results.
- We demonstrated repeatable differentiation on white blood cells using whole blood sample input into a card. The measured counts were with 6% of the measurements made on the same sample using a commercial hematology analyzer (Abbott Cell Dyn 3700).

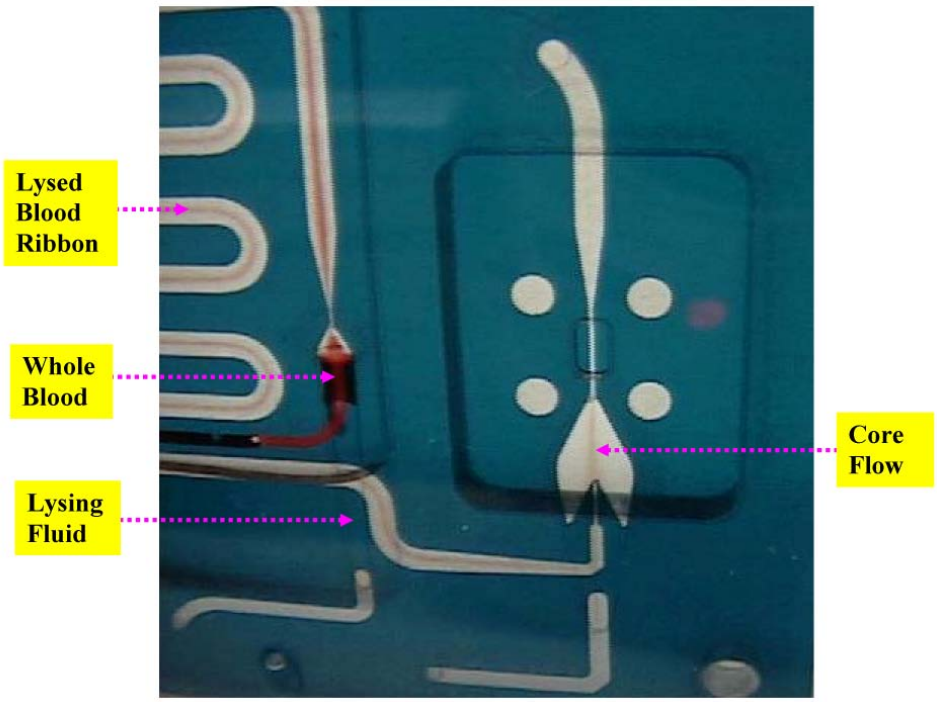


Figure 20. Three channel card showing lyse on the fly operation

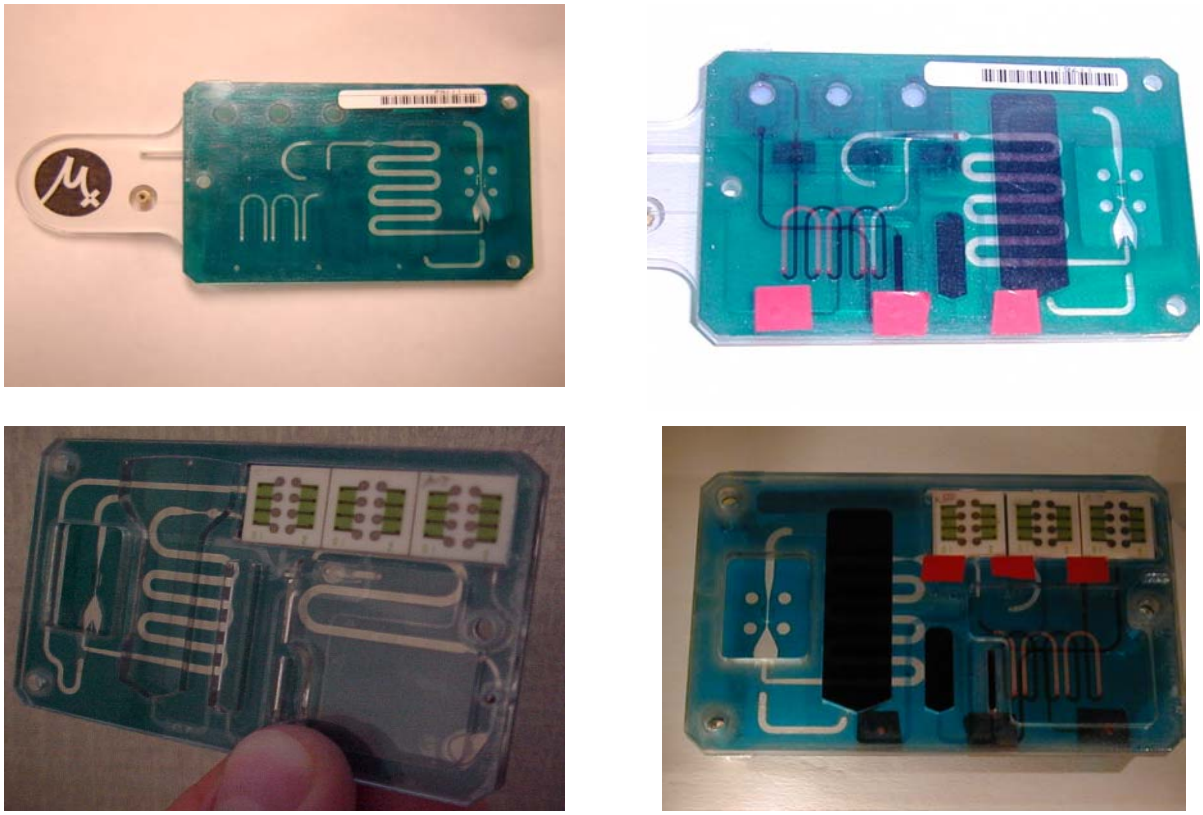


Figure 21. Examples of unfilled and filled (with reagents) fully integrated cards (HWC 28)

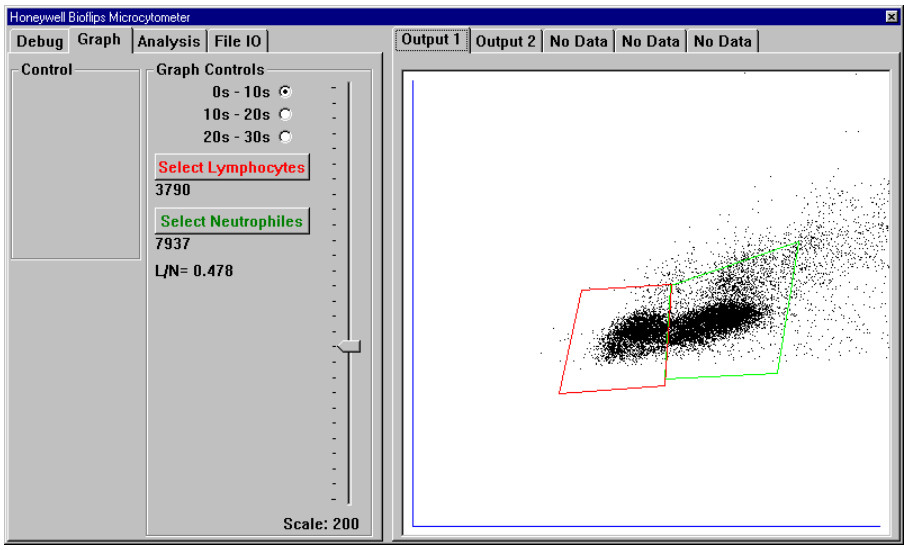


Figure 22. Scatterogram for a two bead mixture showing a measured bead count ratio of 0.478. The expected ratio was 0.5

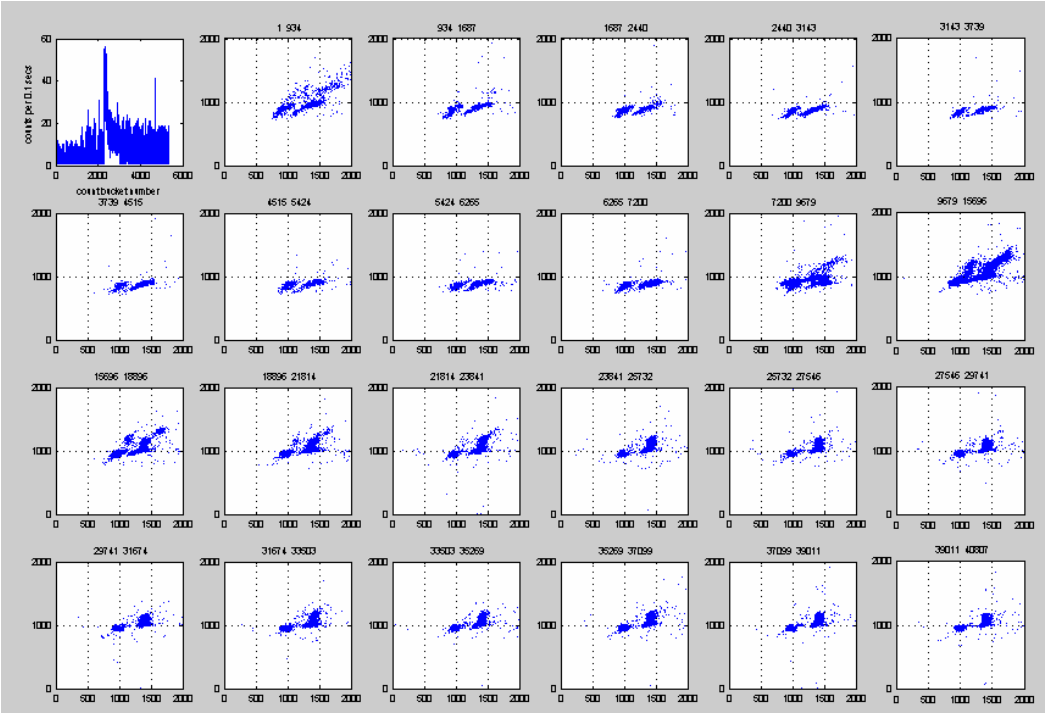


Figure 23. Frame by frame analysis of data during a run that is used for the selection of the "sweet spot" for the instrument operation

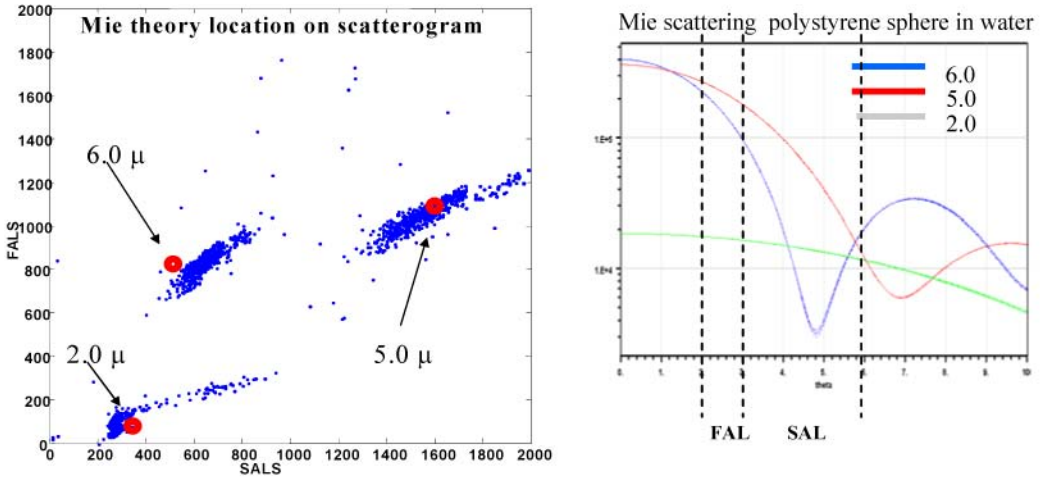


Figure 24. Scatterograms measured for a three bead mixture showing good agreement with theory

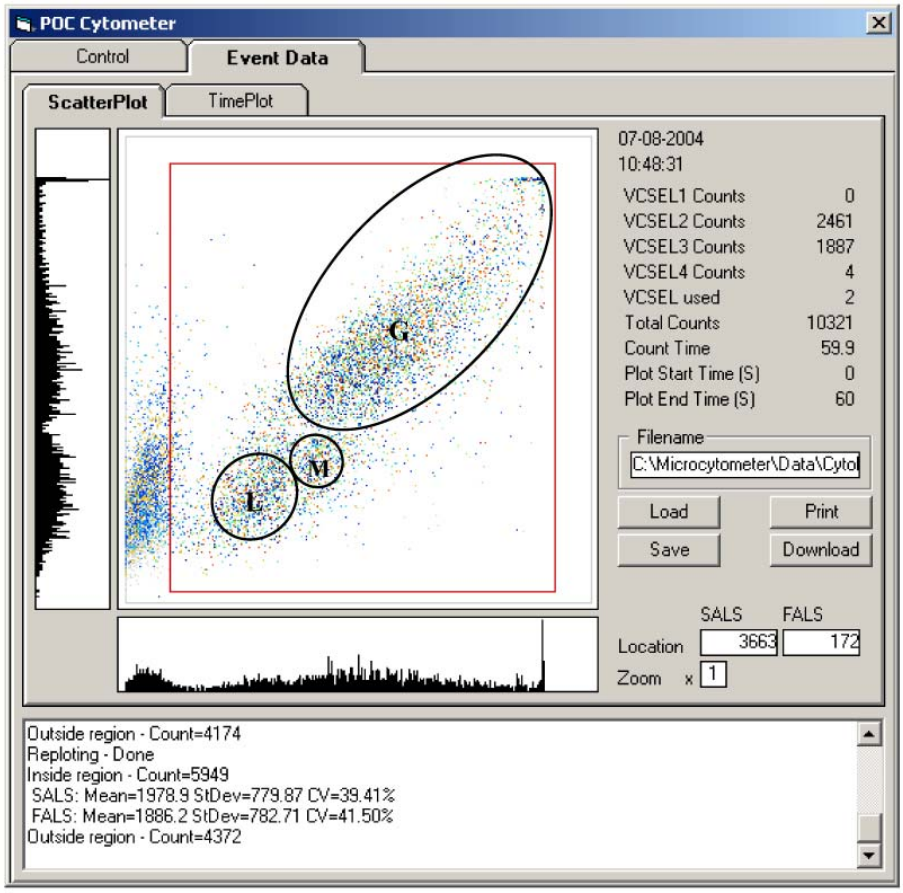


Figure 25. Scatterogram showing 3-part differentiation of white blood cells obtained using a whole sample input into a card and using lyse on the fly operation on the first generation brassboard cytometer

4.2 Fluorescence Cytometer

Fluorescence Flexible testbed

Initial testing was done in a fluorescence testbed format which is pictured in Figure 26 below.

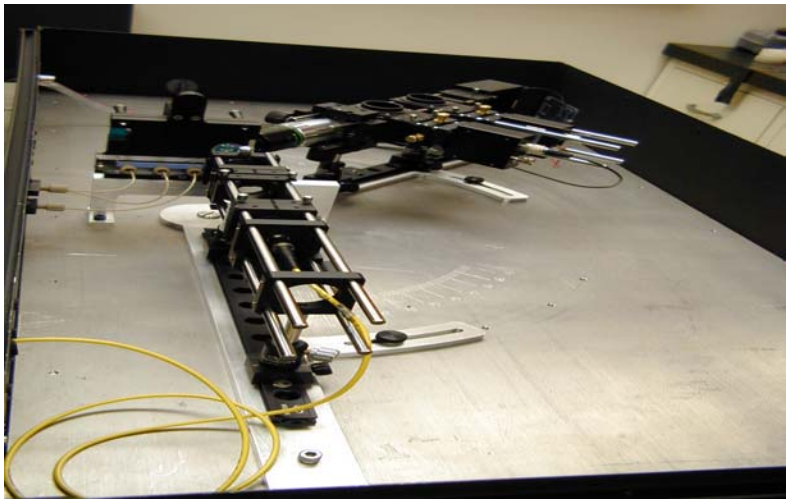


Figure 26. Photograph of Breadboard Fluorescence cytometer, also called testbed

The fluorescence testbed test conditions consisted of a miniaturized pumping system with flow sensors and reagents outside of the cartridge. The source was a 488nm solid state laser and the detectors were PMT's. Optical filters and lenses for working with Alexa Fluor 488 and R-PE dyes were used. A light-tight box was used to improve the signal-to-noise ratio of detection. Both two-channel and three-channel cartridges were used.

Test Results – Fluorescent Bead Mixtures and Whole Blood Testing

Bead data obtained from the testing of fluorescent bead mixtures is shown in figures 27 –29.

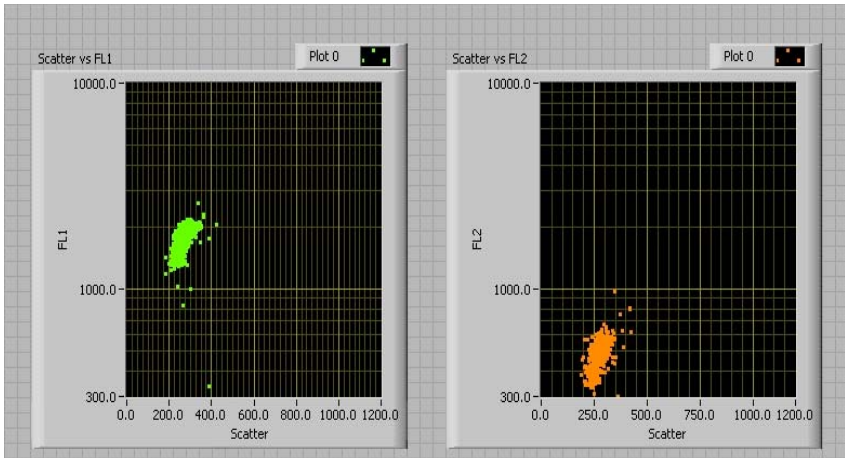


Figure 27: Fluorescent light signal measured on each of the PMT detectors obtained with 6 um diameter bead mixtures coated with Alex Fluor and R-PE dyes. Clear clustering of the fluorescent light can be seen.

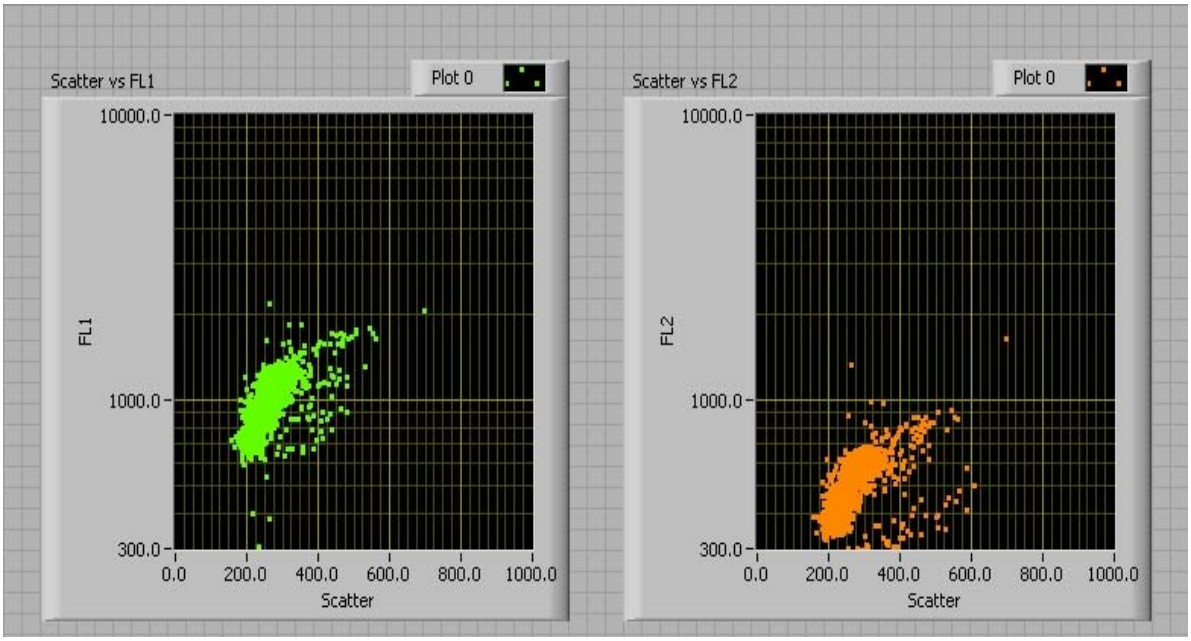


Figure 28: Fluorescent light signal measured on each of the PMT detectors obtained with 6 um diameter bead mixtures coated with Alex Fluor and R-PE dyes. Clear clustering of the fluorescent light can be seen.

The expected count for the above runs was XX and the actual counts was YY.

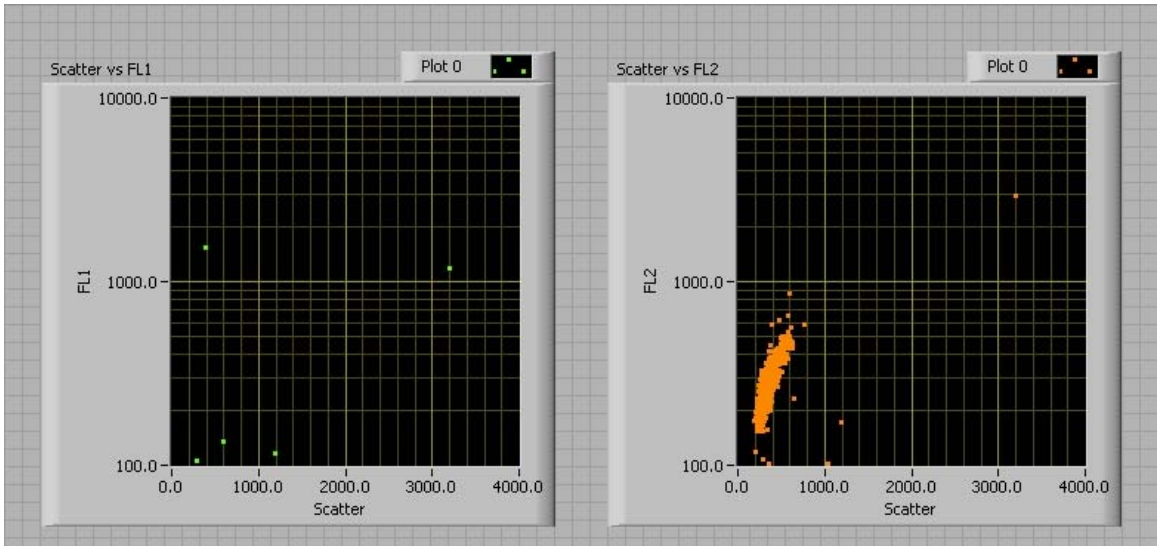


Figure 29: Fluorescent light signal measured on each of the PMT detectors obtained with 6 um diamter bead mixtures coated with only R-PE dye. Clear clustering of the fluorescent light can be seen and no signal is seen on FL1 channel.

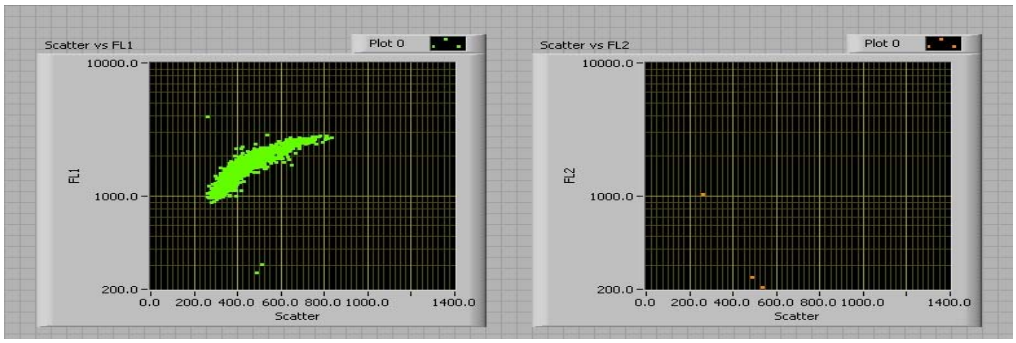
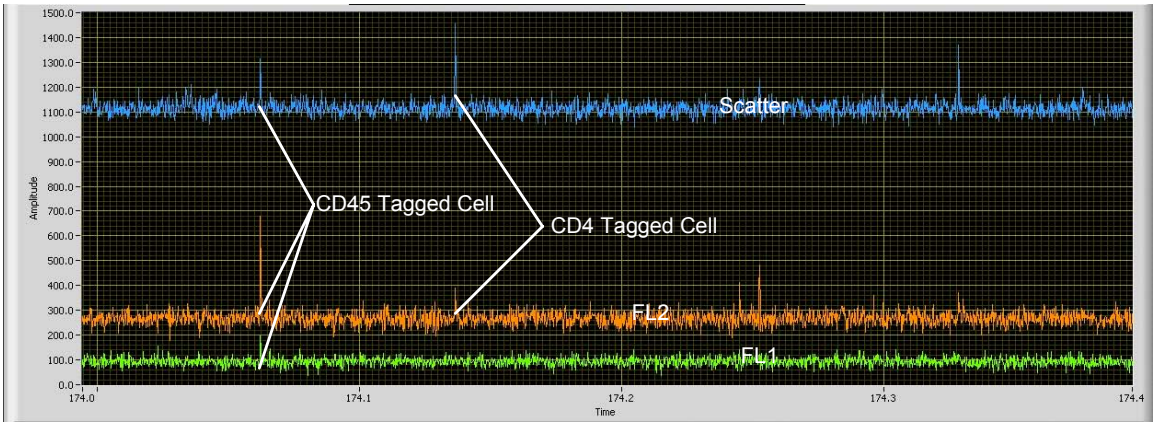


Figure 30: Fluorescent light signal measured on each of the PMT detectors obtained with 6 um diamter bead mixtures coated with only Alex Fluor dye. Clear clustering of the fluorescent light can be seen and no signal is seen on FL2 channel.



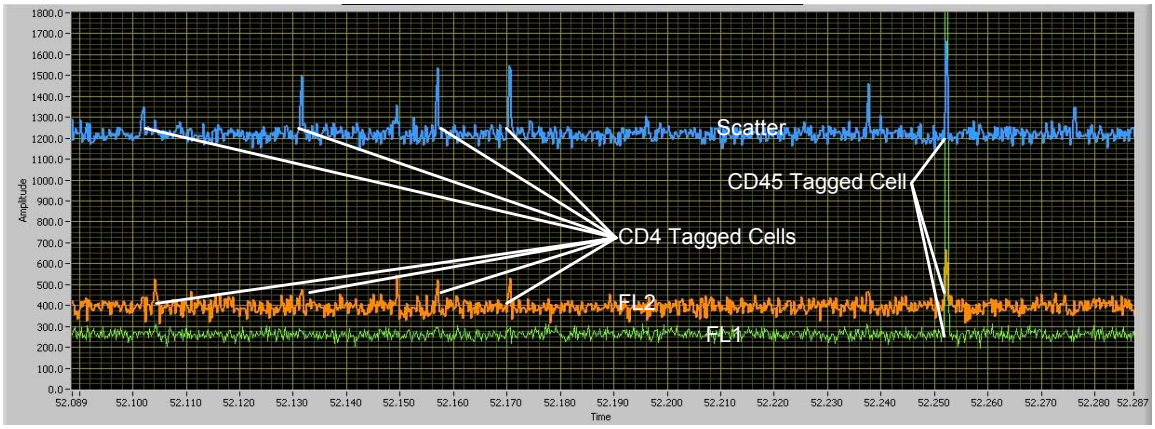


Figure 31: Fluorescent light signal measured on each of the PMT detectors obtained from lysed whole blood tagged with CD4 and CD45 antibodies, as measured in the miniaturized fluorescence cytometer and using a disposable analysis cartridge.

4.3 Cartridge

4.4.1 Scattering Cytometer CartridgeSpecific results achieved during the scattering cytometer cartridge development are as follows.

4.4.1.1 Sample Acquisition Results:

Design	Results	Report
HW CAP-01	Passive capillary action fills sample loop, but filling stops at valve.	"Sample Collector System Report"
HW CAP-02	Added finger feature to via to help sample flow. Sample was able to fill loop (~10-15 µl).	"Sample Collector System Report"
CAP-TEST-02	Capillary fills in 10-15 seconds when plasma treated and no heat aging. Fills in 40-60 seconds when card is head aged. Active positive and negative pressure channels fill in 1 second.	HW2002-0103
CAP-TEST-07	Bellows draws past drum valve which is then shut off. Closing drum valve pushes sample past zero valve into waste. Pressing bellows again empties sample to waste.	HW2002-0103
CAP-16	Sample is pushed into waste using air.	HW2002-0129
CAP-19	Actuate drum valve with manually operated bellows to close off sample from sample reservoir.	HW2002-0129

4.4.1.2 Reagent Storage Fluid Loss :

Design	Storage Type	Fluid Loss	Report
CAP-03	Large (1.5 ml) reservoir	231 µl/week	HW2002-0103
CAP-06	180 µl of fluid storage in a thin fluid channel	7.3±0.8 µl/week	HW2002-0103
CAP-11	1 ml reagent storage in a 1.6 mm thick acrylic (PMMA) channel.	2 µl/day	HW2002-0103
CAP-22	670 µl of fluid in an acrylic channel	12.0±0.3 µl/week	HW2003-0322
HWC-12	Three storage areas: • sheath (1.1 ml), • lyse (0.2 ml) • sample pushing liquids (12.3 µl)	12.6±6 µl/day (Sheath only: 0.6±0.1 µl/day)	HW2003-0708

4.4.1.3 Core Characterization

Modeling estimates versus measurements:

Design	Flow Rate Ratio	Predicted Width (µm)	Measured Width (µm)	Report
20-AL-47-5	350	15.1	16	HW2000-1103
20-AL-47-5	105	17	27	HW2000-1103

Measurements:

Design	Sample Flow Rate	Sheath Flow Rate	Sheath Sample	Core (microns)	STD	Num Tested	Comments	Report
20-AL-54	0.1	2	20	14.8	3.1	6		HW2001-0330
20-AL-54	0.1	4	40	11.6	1.6	14		HW2001-0330
20-AL-54	0.1	4	40	10.9	2.1	6		HW2001-0330
20-AL-54	0.15	6	40	11.9	1.8	14		HW2001-0330
20-AL-54	0.2	8	40	13.2	2.2	14		HW2001-0330
20-AL-54	0.1	6	60	9.4	3.6	6		HW2001-0330
20-AL-54	0.1	8	80	7.4	1.7	6		HW2001-0330
20-AL-54	0.0525	5.25	100	7.6	0.6	3		HW2001-0330
20-AL-54	0.1	10	100	7.2	2.1	6		HW2001-0330
20-AL-54	0.105	10.5	100	14.8	4	8	1-leaker	HW2001-0328
20-AL-54	0.105	10.5	100	11.2	5.7	3		HW2001-0330
20-AL-54	0.03	10.5	350	12.3	5.0	3		HW2001-0330
20-AL-54	0.015	5.25	350	9.9	3.0	3		HW2001-0330
20-AL-54A	0.1	2	20	13.3	3.3	4		HW2001-0330
20-AL-54A	0.1	4	40	11.3	3.7	4		HW2001-0330
20-AL-54A	0.1	6	60	8.5	1.2	4		HW2001-0330
20-AL-54A	0.1	8	80	7.1	1.5	4		HW2001-0330
20-AL-54 5A	0.0525	5.25	100	23.9	5.3	4		HW2001-0328
20-AL-54A	0.0525	5.25	100	13.1	5.0	3		HW2001-0330
20-AL-54A	0.1	10	100	6.6	3.2	4		HW2001-0330
20-AL-54 5A	0.105	10.5	100	23	6.1	4		HW2001-0328
20-AL-54A	0.105	10.5	100	10.5	0.8	3		HW2001-0330
20-AL-54 5A	0.015	5.25	350	20.1	2.1	4	1-pulsing	HW2001-0328
20-AL-54A	0.015	5.25	350	14.0	3.4	3		HW2001-0330
20-AL-54 5A	0.03	10.5	350	15.4	6.5	4	1-pulsing	HW2001-0328
20-AL-54A	0.03	10.5	350	8.9	3.2	3		HW2001-0330
20-AL-54 5B	0.0525	5.25	100	21	2.2	4	1-leaker, 1-pulsing	HW2001-0328
20-AL-54 5B	0.105	10.5	100	18	2.9	4	1-leaker	HW2001-0328
20-AL-54 5B	0.015	5.25	350	21.4	2.6	4	1-leaker, 1-pulsing	HW2001-0328
20-AL-54 5B	0.03	10.5	350	17.3	na	4	3-leaker, 1-pulsing	HW2001-0328
20-AL-54 5C	0.0525	5.25	100	58.6	33.8	4	1-leaker	HW2001-0328
20-AL-54 5C	0.105	10.5	100	56.21	21.3	4	1-leaker, 1-pulsing	HW2001-0328
20-AL-54 5C	0.015	5.25	350	52.2	15	4	1-leaker	HW2001-0328
20-AL-54 5C	0.03	10.5	350	54.8	3	4	1-leaker, 1-pulsing	HW2001-0328
20-AL-54D	0.1	2	20	13.9	1.9	4		HW2001-0330
20-AL-54D	0.1	4	40	14.0	1.6	5		HW2001-0330
20-AL-54D	0.1	6	60	12.1	1.5	5		HW2001-0330
20-AL-54D	0.1	8	80	10.1	1.8	5		HW2001-0330
20-AL-54 5D	0.0525	5.25	100	20.8	14.1	4	1-leaker	HW2001-0328
20-AL-54D	0.0525	5.25	100	9.9	na	1		HW2001-0330

Design	Sample Flow Rate	Sheath Flow Rate	Sheath Sample	Core (microns)	STD	Num Tested	Comments	Report
20-AL-54D	0.1	10	100	9.2	1	5	1-bubble	HW2001-0330
20-AL-54 5D	0.105	10.5	100	19.1	5.3	4	1-leaker	HW2001-0328
20-AL-54D	0.105	10.5	100	12.1	na	1		HW2001-0330
20-AL-54D	0.015	5.25	350	16.0	na	1		HW2001-0330
20-AL-54 5D	0.015	5.25	350	15.7	1.9	4	1-leaker, 1-no core	HW2001-0328
20-AL-54D	0.03	10.5	350	19.8	na	1		HW2001-0330
20-AL-54 5D	0.03	10.5	350	17.2	6.7	4	1-leaker	HW2001-0328
20-AL-54 5E	0.0525	5.25	100	43.4	12.6	4	2-leaker	HW2001-0328
20-AL-54 5E	0.105	10.5	100	40.2	17.8	4	2-leaker	HW2001-0328
20-AL-54 5E	0.015	5.25	350	36.8	11.1	4	2-leaker	HW2001-0328
20-AL-54 5E	0.03	10.5	350	44.5	9.2	4	2-leaker	HW2001-0328
20-AL-54 5F	0.0525	5.25	100	15.1	na	4	3-leaker	HW2001-0328
20-AL-54 5F	0.105	10.5	100	15.3	na	4	3-leaker	HW2001-0328
20-AL-54 5F	0.015	5.25	350	----	na	4	3-leaker, 1-pulsing	HW2001-0328
20-AL-54 5F	0.03	10.5	350	----	na	4	3-leaker, 1-pulsing	HW2001-0328
20-AL-60	0.1	4	40	14.9	1.8	4		HW2001-0330
20-AL-60	0.15	6	40	19.1	8.1	4		HW2001-0330
20-AL-60	0.2	8	40	20.2	7.3	4		HW2001-0330

4.4.1.4 Lysing Subcircuit Measurements—At 0.1 µl/s blood flow, a reasonable core was formed in the injection chamber, but at 0.05 µl/s, clumping occurred in the chamber and the ribbon became very unstable. Thus, a blood flow rate of 0.05 µl/s should be used as an absolute minimum [5].

Design	Sample Flow Rate (µl/s)	Lyse Flow Rate (µl/s)	Distance Required for Lysing (mm)	Report
HW DIF-08	0.1	2.0	33	HW2001-0316
HW DIF-08	0.05	2.0	16	HW2001-0316

4.4.2 Fluorescence Cytometer Cartridge

4.4.2.1 Stain-and-Lyse-on-the-Fly Test Results

- Stain and lysing occurred on Micronics' microFlow station with Honeywell manifold (see Figure 30). Cells transported to commercial cytometer for characterization.

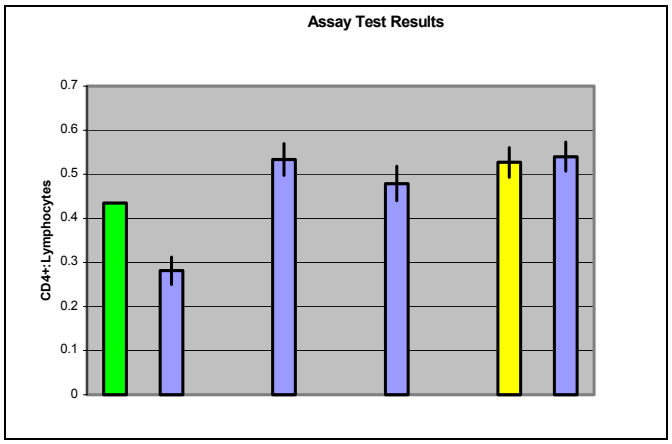


Figure 32. CD4/CD45 Assay Results—Run on Micronics' microFlow and Commercial Cytometer

Section 5. Important Findings and Conclusions

5.1 Scattering Cytometer

The successful operation of the palmsize scattering cytometer required the following:

- Closed-loop pumping of various fluids in order to achieve the timing and precision necessary for performing sample preparation and cytometric analysis
- Electronic self alignment based on red VCSEL arrays allowed the use of low cost cards with dimensional variations.
- Self-contained disposable cards with on-board whole blood sample collector, reagents and liquid flow sensors.

5.2 Fluorescence Cytometer

The successful operation of the fluorescence cytometer required the following:

- High efficiency and high sensitivity detection side optics to enable the detection of low intensity fluorescent signals from tagged antibodies.
- The use of low autofluorescence materials in all aspects of the optical subsystem and on the optical windows on the disposable cards.
- Closed-loop pumping of various fluids in order to achieve the timing and precision necessary for performing sample preparation and cytometric analysis
- The use of self-contained disposable cards with on-board whole blood sample collector, and antibodies.

5.3 Cartridge

As noted throughout this report, the development of a complex microfluidics-based lab card for target applications ranging from white blood cell differential analysis to rapid CD4/CD45 counting required the ability to:

- Work directly from complex real world sample (e.g., whole blood);
- Eliminate all sample processing and devise integrated circuits on card to process the complete assay of interest;
- Assess materials (plastics and adhesives), treatment methods, and test procedures to troubleshoot design issues, both on-card and card-instrument related.

The integrated three dimensional lab card, which it produced using manually intensive prototype methods, was shown to achieve the target objectives, but this method of production would not be acceptable for a commercial product. Lot-to-lot variability and the challenges of scaling up

volume production in a hand-tooled device are best addressed in subsequent development effort using tooling and ultimately injection mold methods.

Results obtained comparing the CD4/CD45 card to commercial cytometry clearly reflect the benefit of continued advancement of a microfluidics-based lab card. The ability to greatly reduce labor, time, reagents, and sample volume makes this a readily deployable approach in conjunction with a portable or benchtop detection platform.

Section 6. Significant Hardware Development

6.1 Instrument

6.1.1 The Breadboard Microcytometer (α-Platform)

A flow cytometer was designed based on a miniaturized disposable fluidic chip of roughly 8 x 5 cm^2 demonstrating the VCSEL-based detection approach for flow cytometry. The sample collector and reagent fluids are external to the fluidic card (Month 12).

6.1.2 The First-Generation Brassboard Prototype Microcytometer (Version 1.0)

A miniaturized palmsize flow cytometer with reagent fluids & flow sensors located on the instrument. The miniaturized disposable fluidic card has an integrated sample collection system. Custom VCSEL array, detector, and closed-loop flow controller were integrated to yield a compact measurement system (Month 24). Blood cell counting and identification was demonstrated with this instrument.

6.1.3 The Second-Generation Brassboard Prototype Microcytometer (Version 2.0)

A miniaturized palmsize flow cytometer with reagent fluids & flow sensors located on the disposable card. (Month 36). The instrument is fully dry and all wet fluidic processing is done on the card. Whole blood core formation and lyse on the fly operation was demonstrated on this instrument.

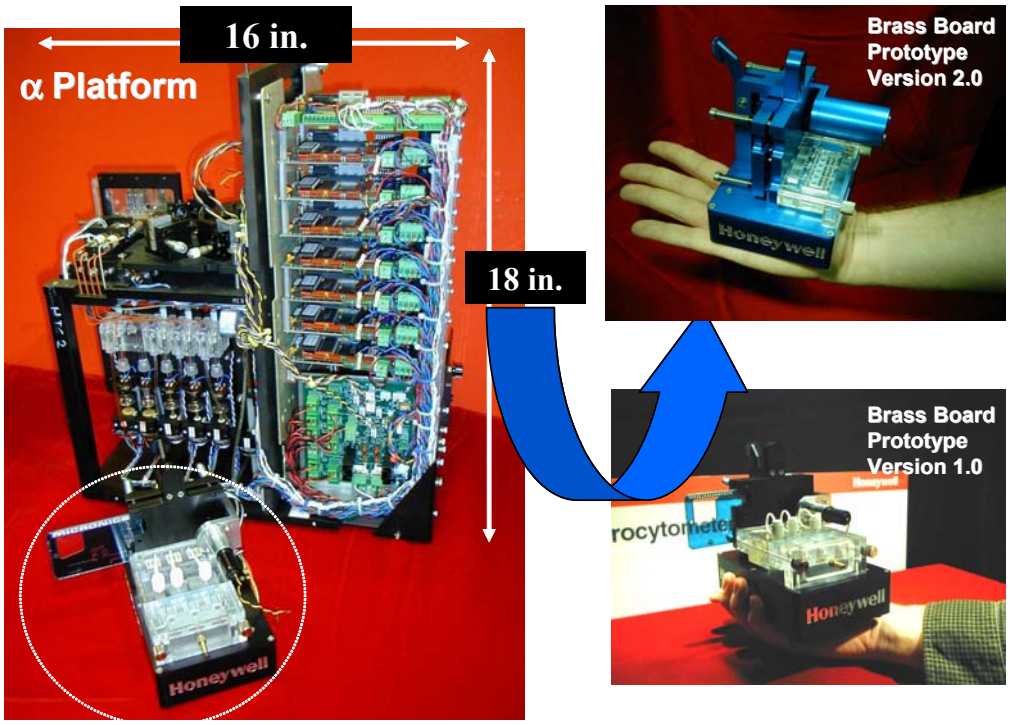


Figure 33. Breadboard (Alpha Platform), Brassboard Cytometers

Figure34. Brassboard cytometer with handheld PC

6.1.4 The Integrated Scattering and Fluorescence Cytometer

A miniaturized flow cytometer with reagent fluids & flow sensors located on the instrument and capable of both light scattering and light fluorescence based measurements. (Month 48). Counting & classification of two color bead mixtures was demonstrated.

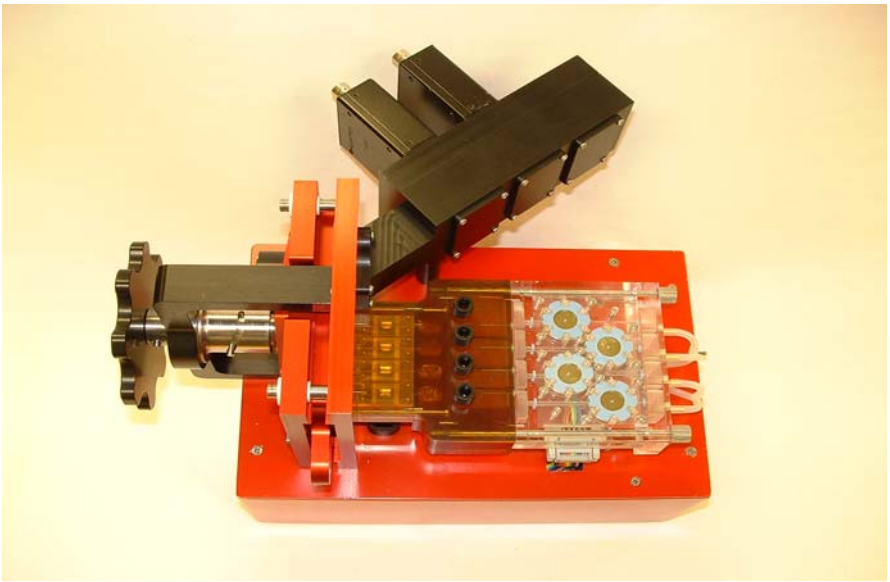


Figure 35. Integrated Scattering and Fluorescence Cytometer

6.2 Cartridge

The following type of cartridge hardware was built as part of this project:

- Two channel cards (HWC 22)– no reagents or flow sensors on card. No sample preparation capability on card. Useful for counting prepared samples.
- Three channel cards (HWC 26) – no reagents or flow sensors on card. Sample preparation capability on card. Useful for doing lyse on the fly operation of whole blood on card.
- Fully Integrated Card (HWC 28) – All reagents and three flow sensors on card. Sample preparation capability on card. Can do lyse on the fly operation of whole blood on card.
- Fluorescnce Two Channel Card - no reagents or flow sensors on card. No sample preparation capability on card. Useful for counting fluorescent bead mixtures.
- Fluorescence Fully Integrated Card – Antibodies on card but no flow sensors on card. Sample preparation capability on card. Useful for doing lyse on the fly and stain on the fly operation of whole blood on card.

Photographs & schematic drawings of the various card types are given in various sections of this report.

Section 7. Special Comments

7.1 Scattering Cytometer

None.

7.2 Fluorescence Cytometer

None.

7.3 Cartridge

None.

Section 8. Implications for Further Research

8.1 Instrument

Further effort in the following areas (some of which are on going withing Honeywell) will enable the rapid commercialization of this technology for both military and commercial applications.

- Improve reliability of key components to allow for volume manufacture and field use of the instrument
- Perform statistically relevant number of measurements on normal, abnormal blood samples to clearly establish clinical significance of the measurements.
- Modify the two-color fluorescence cytometer to allow the use of environmental (as opposed to blood) samples for the detection biowarfare agents. The team expects to submit technology transfer proposals to the military for this application area.

8.2 Cartridge

- Further effort is required to improve quality. The current Micronics lamination approach for card manufacture is a flexible approach allowing the team to change card designs easily but is not suited for volume production. Card quality issues in the later stages of the program prevented the team from making statistically large number of repeat measurements. In follow-on technology transition programs the team will explore alternative manufacturing approaches to improve card yield and allow for the development of a fieldable instrument.
- Once the instrument-card interface has been established to work routinely and robustly for given target applications, commercial card production (e.g., tooling and injection molding) processes will need to be addressed and refined. The team has initiated efforts with regard to commercial card development, including but not limited to on-card reagent optimization, packaging, and labeling, as well as regulatory compliance and filings.

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